1,5 iodonaphthyl Azide-Inactivated V3526 Protects against Aerosol Challenge with Virulent Venezuelan Equine Encephalitis Virus.

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Abstract:

Venezuelan equine encephalitis virus (VEEV) is a New World alphavirus. There is no licensed vaccine for prophylaxis against VEEV. VEEV is highly infectious in aerosolized form and has been identified as a bio-terrorism agent. The current IND vaccine is poorly immunogenic and does not protect against an aerosol challenge with virulent VEEV. We have previously shown that VEEV inactivated by 1,5 iodonaphthy azide (INA) protects against footpad challenge with virulent VEEV. In this study, we inactivated an attenuated strain of VEEV, V3526, with INA and evaluated its protective efficacy against aerosol challenge with virulent VEEV. We demonstrated that among three routes of immunization, intramuscular immunization with INA-inactivate V3526 provided complete protection against aerosol challenge with virulent VEEV. Our data suggests that INA-inactivated V3526 may be explored further for specific development as an effective vaccine candidate against aerosol challenge of virulent VEEV.

Highlights:

- INA-iV3526 provided complete protection against aerosol challenge.
- Immunization with INA-iV3526 triggered a strong antibody response.
- IM route of immunization provided maximum amount of protection.

Key words: Venezuelan equine encephalitis, vaccine, inactivated, iodonaphthyl azide, aerosol challenge, and protection.

1. Introduction:

Venezuelan equine encephalitis virus (VEEV) vaccine development has been marred by failure of inactivated vaccines to develop efficient protective response as well as residual virulence associated with the live attenuated vaccine candidates [1]. VEEV is also a bio-threat agent, thereby; any VEEV vaccine should ideally be able to protect against the aerosol challenge with virulent VEEV [2]. Live-attenuated TC-83 vaccine, which is currently used under investigational new drug (IND) status, and its formalin-inactivated form (C-84) provide incomplete protection against aerosol challenge in non-human primates (NHP) [3]. Several other approaches of developing vaccine candidates against VEEV have been tried, but none of these have advanced for development as VEEV vaccine candidate [1, 2, 4].

V3526 is a live-attenuated strain of VEEV that was shown to be safe in animals and environmental hazard studies and provided excellent immunogenicity [5, 6]. However, its development as a vaccine candidate was stopped due to causation of several adverse reactions in the vaccinees during the clinical trial [7]. We have previously shown that V3526 can be completely inactivated by 1,5 Iodonaphthyl azide (INA) as tested by serial passages *in vitro* and by intracranial inoculation in neonatal mice [8]. In this study, we evaluated the protective efficacy of INA-inactivated V3526 (INA-iV3526) against aerosol challenge with virulent TrD strain of VEEV (VEEV-TrD). Three different routes of immunization intranasal (IN), intramuscular (IM), and subcutaneous (SC) were evaluated. Our results demonstrated that INA-iV3526 provides complete protection from aerosol challenge with VEEV-TrD when vaccinated by IM route and therefore, is a strong candidate for further development as a VEEV vaccine.

2. Methods:

Inactivation of V3526: INA was kindly provided by Dr. Robert M Blumenthal, National Cancer Institute, Frederick, MD under a material transfer agreement with Uniformed Services University of the Health Sciences, Bethesda, MD. Sucrose gradient purified V3526 was inactivated by INA (INA-iV3526) as described previously [8]. Briefly, under reduced light conditions, 500 µg of viral protein was mixed with 20 mM INA to achieve a final concentration of 100 µM INA. Samples were then incubated at room temperature for 20 min followed by centrifugation at 1000 rpm for 1 min to remove any precipitated INA. Glutathione was then added to the supernatant to obtain a final concentration of 20 µM. This mix was then exposed to long wavelength UV using a 100W UV lamp for 5 min with intermittent vortexing. The samples were stored at -80°C until further use. Formalin-inactivation of V3526 (FiV3526) was performed by treating with 0.1% v/v formalin at 37°C for 24 hours as described previously [9].

Immunization and challenge procedures: Three different routes of immunizations were tested, intranasal (IN), intramuscular (IM) and subcutaneous (SC). Animals (n= 10/group) received a primary immunization on Day 0 followed by a booster on Day 28. Two immunization doses were tested for each of the inactivated viruses (*i.e.,* 1 and 3µg of either INA-iV3526 or FiV3625

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for IN and SC route of immunization. For IM route, 3 and 5 µg of either INA-iV3526 or FiV3625). For IN immunization, 10 µl of inactivated virus suspension was inoculated in each nostril to achieve a total immunization dose of 1 or 3 µg. For SC immunization, 10 µl of inactivated virus suspension was inoculated under the skin over the neck. Formalin-inactivated TC-83 (C-84) was included as a positive control and administered as routinely given via SC inoculation. For IM immunization, 50 µl of inactivated virus suspension was inoculated in the thigh muscle of the hind leg. Mock immunization was done with equal volumes of saline for each route of immunization. Serum was collected by retro-orbital bleed from animals in all the groups on Day 49 after the first immunization. All the animals were challenged with VEEV Trinidad strain (VEEV-TrD) by aerosol route as described earlier [9]. Spray factors and inhaled dose for each of the group is given in supplementary table 1. Mice were challenged 4 weeks after the last immunization.

Ethics Statement: Animal experiments involving aerosol challenge with infectious VEEV-TrD were carried out in ABSL-3 containment facility at United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory animals, National Research Council, 2011.

Microneutralization assay: Serum samples were analyzed for neutralizing antibody response by microneutralization test (MNT80) (Grund et al 2011 ref). Briefly, Vero 76 cells were seeded at a density of 4 x 10^4 cells/well of a 96-well plate (Corning, VWR Cat #29443-050, Bridgeport, NJ) and incubated overnight at 37^oC, 5% CO_2. Serial dilutions of serum samples were prepared in Hank's Buffered Saline Solution (HBSS). Virus (125 PFU/well) was added to diluted serum samples and incubated overnight at 4^oC. Following incubation, sample+virus mixtures were transferred to a 96-well plate containing Vero cells and incubated for 24 h at 37^oC, 5% CO_2. After 24h, the cells were fixed with 10% formalin for 24h at 4^oC. After fixing, infected cells were labeled using a monoclonal antibody to the E2 glycoprotein (mAb 1A4A, 1:4000) followed by secondary antibody, horseradish peroxidase-labeled goat-anti-mouse (KPL Laboratories, 1:10,000). Detection of cells expressing VEEV E2 protein utilized ABTS substrate (KPL Laboratories, Gaithersburg, MD) incubated at RT for, 20 min at which time optical density (OD) at 410 nm was determined using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). Endpoint titers were determined as the highest dilution that produced an OD greater than the mean OD of the negative controls wells plus 3 standard deviations.

Serum IgG ELISA: Briefly, Costar EIA/RIA 96-well high-binding plates (Corning Inc., Corning, NY) were coated with 0.2 µg of sucrose purified VEEV Trinidad Donkey strain (VEEV-TrD) per well and incubated overnight, or up to 1 week, at 4^oC. The following day, plates were

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blocked with Dulbecco's phosphate buffered saline (DPBS) (GIBCO[TM] Invitrogen Corp., Grand Island, NY) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) and 5% nonfat dry milk (Becton Dickinson and Co., Sparks, MD) (PBSTM) for 2 hours at 37°C. The plates were washed 3 times with PBST using the BioTek ELx405[TM] microplate washer (BioTek Instruments, Inc., Winooski, VT). Mouse sera were diluted in PBSTM containing 1% heat inactivated fetal bovine serum (GIBCO[TM] Invitrogen Corp., Grand Island, NY), added to the plate and serially diluted 1:2 and then incubated for 1-2 hours at 37°C. Plates were washed 3 times with PBST followed by the addition of peroxidase-labeled goat anti-mouse IgG 1:50,000 (KPL, Gaithersburg, MD). The plates were incubated with the secondary antibody for 1 hr at 37°C and then washed 3 times with PBST. The ABST Peroxidase substrate (KPL, Gaithersburg, MD) was added to each well and color developed for approximately 20-30 min at which time the OD at 410 nm was determined using a Spectramax M5 microplate reader (Molecular Devices). The negative control was normal mouse serum (Sigma Aldrich, St. Louis, MO) and was diluted 1:100. The positive control was pooled serum from previously vaccinated adult mice which survived challenge with parental virus and was diluted 1:400. Endpoint titers were determined as the highest two-fold dilution that produced an OD greater than the mean OD of the negative controls wells plus 3 standard deviations.

3. Results:

IN immunization with INA-iV3526 provided poor protection against aerosol challenge with VEEV-TrD. Immunization with 1 µg of INA-iV3526 did not provide any protection against the virulent challenge and all the animals succumbed to the challenge with virulent VEEV. Immunization with 3 µg of INA-iV3526 protected only 10% of mice from the virulent VEEV challenge. In contrast immunization with 1 and 3 µg of FiV3526 provided 60 and 70% protection, respectively, from the virulent VEEV challenge. As expected all the mock-immunized mice succumbed or met euthanasia criteria following infection with virulent VEEV exposure (Figure 1A). All mice lost weight initially, but those who survived exposure to virulent VEEV-TrD started gaining weight around 6-7 day post-challenge. However, average weight gain in the 3 µg INA-iV3526 immunized group was lower than that of the FiV3526 immunized groups (Supplementary Figure 1A).

SC immunization with INA-iV3526 significantly protected mice against aerosol challenge with VEEV-TrD. Immunization with either 3 or 5 µg of INA-iV3526 protected 90% of the animals from the virulent VEEV challenge. This protection was similar to FiV3526 which also protected 90% of mice from challenge with virulent VEEV at both 1 and 3 µg dose of immunizations. As previously demonstrated (Hart 1997 Vaccine 15:363 ref), C-84 protected only 30% of the immunized mice from aerosol challenge with virulent VEEV (Figure 1B). Animals across all immunization groups initially lost weight after challenge with VEEV-TrD, followed by weight gain 6-7 days after the challenge (Supplementary Figure 1B).

IM immunization with INA-iV3526 provided complete protection against aerosol challenge with VEEV-TrD. Immunization with either of the doses of 3 or 5 µg of INA-iV3526 conferred 100% protection from challenge with the virulent VEEV-TrD. In contrast, FiV3526 at doses of 3 and 5 µg protected only 60% and 80% of the immunized mice, respectively. As expected all mock immunized mice succumbed or met euthanasia criteria following challenge with virulent VEEV (Figure 1C). Again animals across all the immunization groups lost weight initially after challenge with virulent VEEV-TrD and then steadily gained weight from approximately 6 day onward(Supplementary Figure 1C).

Neutralizing antibody and total serum IgG titers were measured at day 49 after primary immunization. Overall, high total serum IgG titers correlated with protection. In IN immunization experiments, neutralizing antibodies and total serum IgGs were not detected in the INA-iV3526 immunized mice which little to no protection from the virulent TrD challenge (Figure 2 A and 3A). In SC immunization experiments, where 90% protection was observed in both FiV3526 and INA-iV3526 immunized animals, neutralizing antibody titers in INA-iV3526 group were lower than the FiV3526 groups. Surprisingly, neutralization titer in C-84 immunized group which provided poor protection from aerosolized VEEV-TrD challenge, was higher than the INA-iV3526 immunized groups (Figure 2B). Total serum IgG were of similar levels among all the immunization groups (Figure 3B). In IM immunization experiments, neutralizing antibody titers in INA-iV3526 immunized groups were lower than that of the FiV3526 group (Figure 2C). Total serum IgG titers, however, were similar among all the IM immunized groups (Figure 3C).

4. Discussion:

Previously, we reported that V3526 can be completely inactivated using INA in the presence of UV-irradiation [8]. In this study, protective efficacy of INA-inactivated V3526 against aerosol challenge with virulent VEEV-TrD was demonstrated. V3526 is a furin-cleavage mutant of V3000 (full length clone of VEEV-TrD) and induces excellent immunogenicity, but caused adverse reaction in the vaccinees during the clinical trials [5-7]. Therefore, efforts to further develop V3526 as vaccine candidate were stopped. Inactivation of V3526 by INA was undertaken to impart another layer of safety over an already attenuated virulence profile. INA is a hydrophobic compound which covalently binds to the lipoproteins in the viral envelope without affecting the structural integrity of the surface epitopes [10-12]. Several enveloped viruses including VEEV, and a non-enveloped virus have been shown to be inactivated by INA, while retaining their immunogenic properties [10, 11, 13-18]. Due to the bioweapon/bioterrorism potential of VEEV, a countermeasure that can protect against aerosol exposure is desired [19]. The current IND vaccines (TC-83 and C-84) provide only partial protection against aerosol challenge with virulent VEEV [3]. Among the three different route of vaccination tested in this study, IM route provided 100% protection from aerosol challenge with VEEV-TrD. These results corroborate an earlier vaccination study with gamma-radiation inactivated V3526 which when mixed with adjuvants, showed protection from aerosol challenge with VEEV-TrD [20]. FiV3526, at low doses of immunization, provided partial protection against aerosol challenge

with VEEV-TrD [9]. In this study, a higher dose of FiV3526, provided increased, but still incomplete, protection using similar immunization regimen and IM vaccination. SC immunization with γ-radiation inactivated- or formalin inactivated-V3526 was shown previously to provide 90% protection against aerosol challenge with VEEV-TrD [9, 20]. In this study, even with increased doses of vaccination, inactivated V3526, either by formalin or by INA, provided only 90% protection. Therefore, although protection provided by SC vaccination with inactivated V3526 was significant, SC route may not provide complete protection against aerosol challenge with virulent VEEV. Of the three routes tested in this study, IN vaccination provided the lowest level of protection against aerosol challenge with VEEV-TrD. This may be due to poor mucosal immunity due to lack of virus replication or recognition of INA-iV3526 by the olfactory epithelium resident immune cells. This was evident by lack of neutralizing and total virus-specific IgG response in mice immunized via IN route with INA-iV3526. A previous study with the live-attenuated V3526 vaccine reported that virus-specific IgA correlated well with the survival after challenge with aerosolized virulent strain of VEEV [21]. Although IgA measurements were not performed in this study, the absence of neutralizing and total serum IgG suggests either a lack of or a poor mucosal response after IN immunization. A similar observation was report for Eastern equine encephalitis virus (EEEV), where intranasal inoculation of INA-inactivated EEEV did not protect against challenge with the virulent EEEV [22]. One surprising observation was that the total and neutralizing serum antibody responses in the INA-iV3526 vaccinated mice were lower than those of mice vaccinated with the formalin-inactivated V3526 by SC or IM routes. The protection conferred by SC or IM vaccination with INA-inactivated V3526 was similar to that of the formalin-inactivated V3526. These results are confounding as neutralizing antibodies are conventionally believed to confer protection from alphaviruses [23, 24]. However, plausible explanations for this result may be that INA-iV3526 preserved an important viral envelope epitope that conferred protection even at low titers such that the quality of responses was improved. Alternatively, it is possible that INA-iV3526 induced an efficient T-cell response which has been suggested to protect mice from challenge with virulent VEEV [25, 26]. Neutralizing and total IgG titers in mice vaccinated with C-84 were greater, similar to that of the INA-inactivated V3526, but still provided less protection against aerosol challenge with VEEV-TrD. This observation was noted previously with C-84, where high neutralizing and total antibody titers did not translate into protection against aerosol challenge with virulent VEEV [9, 20].

In conclusion, INA-iV3526 conferred complete protection against aerosol challenge with virulent VEEV-TrD following IM vaccination presenting a case for further evaluation of INA-inactivated V3526 as a potential VEEV vaccine candidate.

representative of those of the Uniformed Services University of the Health Sciences (USUHS), USAMRIID, the Department of Defense (DoD); or, the United States Army, Navy, or Air Force.
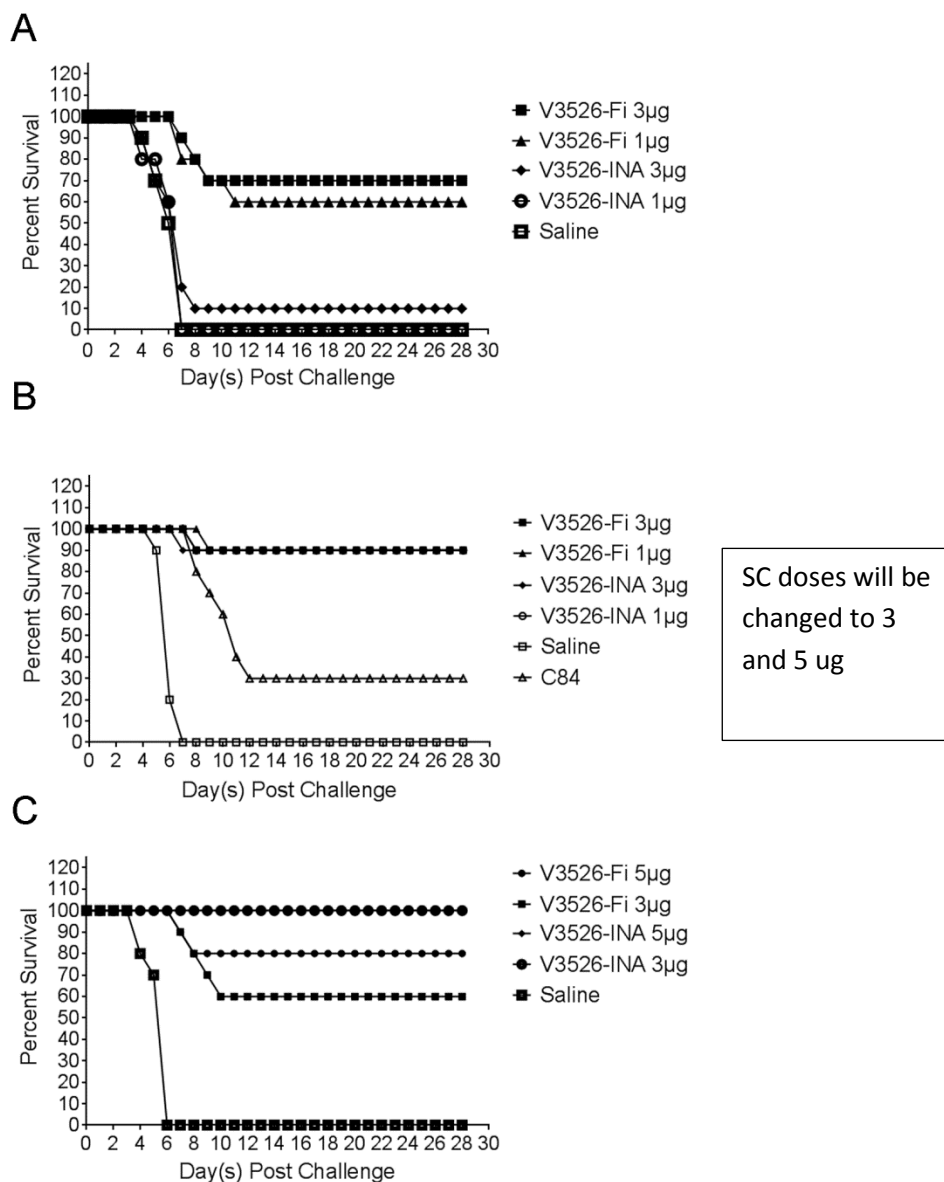
Figures:

Figure 1. Protective efficacy of INA-iV3526 against aerosol challenge with TrD: Mice were immunized with INA-iV3526 or FiV3526. Four weeks after the second immunization, animals were exposed to aerosolized VEEV-TrD. Overall FiV3526 conferred better protection as compared to INA-iV3526 when immunization was provided by IN route (A). Protection conferred by INA-iV3526 and FiV3526 were similar and 90% animals survived the challenge

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when immunization was done by SC route (B). Most protection was observed with IM route of immunization where all the mice immunized with INA-iV3526 survived the challenge whereas FiV3526 conferred maximum of 80% protection with 5 µg dose (C).
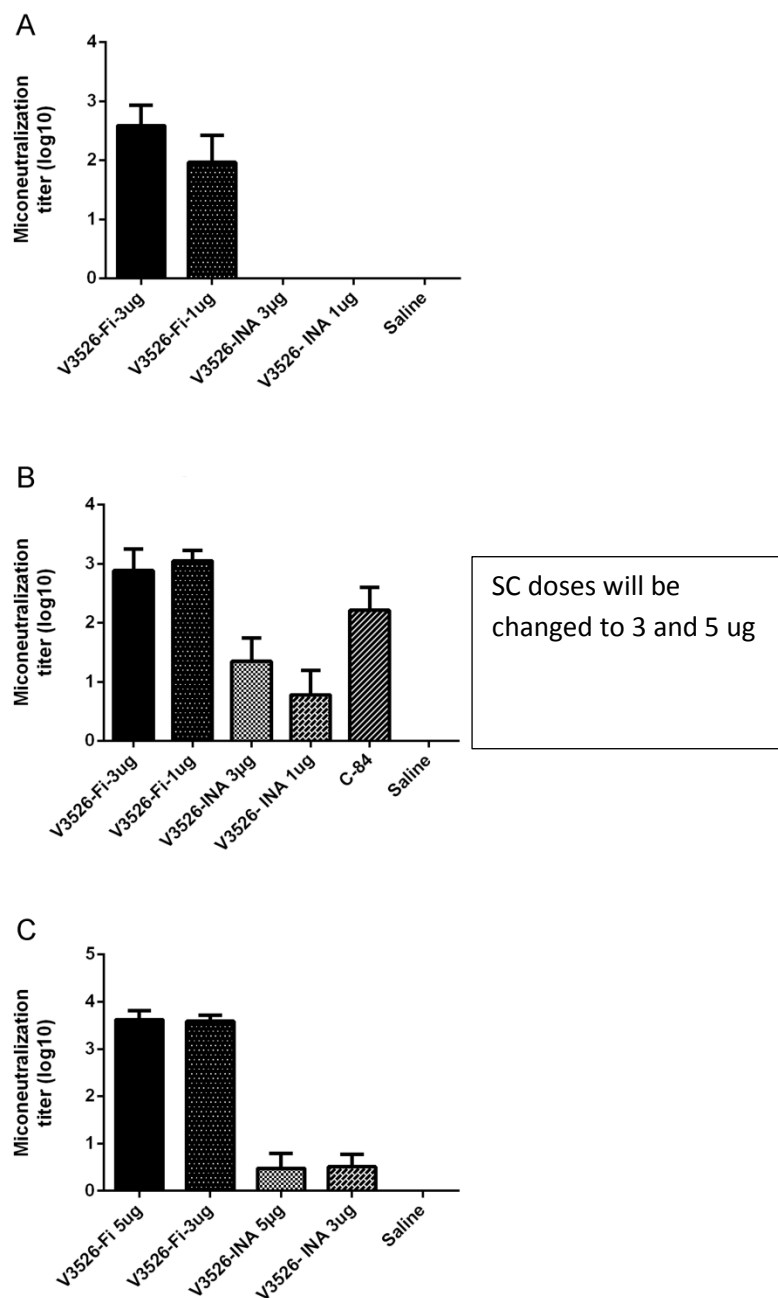
Figure 2:

Figure 2. Neutralizing antibody titers: Neutralizing antibody titers were determined micro neutralization assay. Positive titers were determined as titers that were 2 times that of the

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standard error of the mock controls. (A) IN immunization; (B) SC immunization; and (C) IM immunization. Values are presented as ± SEM.
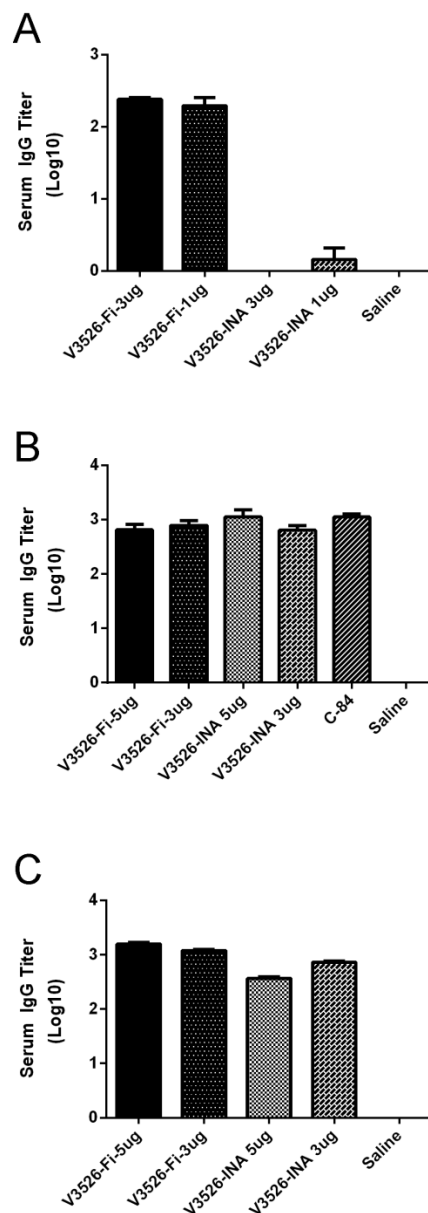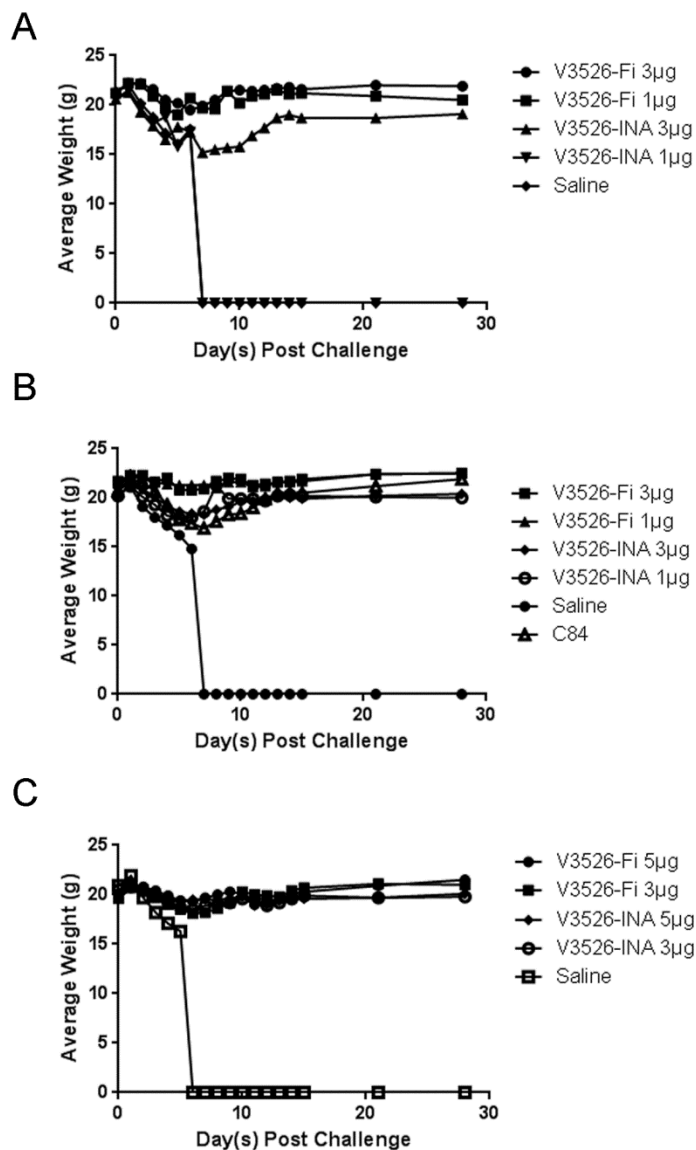
Figure 3:

Figure 3. Serum IgG titers: Total serum IgG titers were determined by ELISA. Positive titers were determined as titers that were 2 times that of the standard error of the mock controls. (A) IN immunization; (B) SC immunization; and (C) IM immunization. Values are presented as ± SEM.

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Figure 1:

Supplementary

SC doses will be changed to 3 and 5 ug

Supplementary Figure 1. Weight gain profile of the immunized mice after aerosol challenge with TrD: Weight of mice were monitored for 28 days after the challenge with measurements taken each day for 2 weeks and after that weight measurement were taken once a week. Overall a slight reduction in body weight was observed in all the immunization groups across the route of immunization. As expected all the mice in saline groups across the route of immunization died by 6-7 day posy challenge. (A) IN immunization; (B) SC immunization; and (D) IM immunization. Values are presented as Average body weight of the live animals in the group.

Supplementary table 1: TrD Aerosol Challenge Summary.

Route	Challenge day	Dose inhaled (pfu/mouse)	Spray factor
IN	d56	454	1.18E-07
IM	d56	897	2.33E-07
SC	d56	555	7.91E-08